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*Original Article*

# Expression of vascular endothelial growth factor C and anti-angiogenesis therapy in endometriosis

Wei-Wei Song[1,2*], Huan Lu[2*], Wen-Jing Hou[2], Guang-Xu Xu[2], Ji-Hong Zhang[2], You-Hua Sheng[2], Ming-Jun Cheng[3], Rong Zhang[2]

*[1]Medical College of Soochow University, Shanghai 215123, P. R. China; [2]Department of Obstetrics and Gynecology, Shanghai Jiaotong University Affiliated Sixth People Hospital South Campus, Shanghai 201499, P. R. China; [3]Obstetrics and Gynecology Hospital, Fudan University, Shanghai 200011, P. R. China. [*]Equal contributors.*

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**Abstract:** Angiogenesis is an important pathogenesis of Endometriosis. Vascular endothelial growth factor C (VEGF-C) is one of the most important factor in the regulation of both normal and abnormal angiogenesis. Anti-angiogenic treatment of endometriosis is still in the exploratory stage. In this study, we investigate the relationship between VEGF-C and endometriosis, the therapeutic effects of Endostar in the rat endometriosis model. We then demonstrated that Immunohistochemical expression of VEGF-C was higher in endometriotic tissues than in control normal ovary tissues ($P < 0.01$) and higher in the endomertriosis grade III-IV than in endomertriosis grade I-II ($P=0.013$). In rat endometriosis model, we observed a significant reduction in the mean volume and weight of the endometriotic implants per rat in the treatment group as compared with the control group. By immunohistochemical evaluation, there was a significant reduction in VEGF-C expression after treatment in all areas examined. VEGF-C may be involved in the pathogenesis of endomertriosis by regulating the angiogenesis. Endostar has therapeutic effects of endometriosis lesions in the rat endometriosis model.

**Keywords:** Endomertriosis, VEGF-C, angiogenesis, rat, Endostar

## Introduction

Endometriosis is a chronic estrogen-dependent disease, affecting 5-10% of women in reproductive age [1, 2]. The pathogenesis of endometriosis is very complex and it has a high recurrence rate. The reported recurrence rate was high, estimated as 21.5% at 2 years and 40-50% at 5 years including dysmenorrhea and lesions reproduction [3]. Endometriosis commonly develops possibly because of large quantities of backwashed menstrual tissue that has become implanted on pelvic organs, primarily on the pelvic peritoneum and ovaries [4]. The common histologic features are the presence of endometrial stromal or epithelial cells, chronic bleeding, and signs of inflammation. The inflammation involved in endometriosis can stimulate nerve endings in the pelvis and thereby cause pain, impair the function of uterine tubes, decrease receptivity of the endometrium, and hinder development of the oocyte and embryo [5, 6]. All of these eventually lead to infertility. Recent studies show that endometriosis is a angiogenesis-dependent disease [7]. The increasing of angiogenic factor activity or decreasing of angiogenesis inhibitor activity is an important factor in the process of the formation of endometriosis [8].

Vascular endothelial growth factor C (VEGF-C) is a member of VEGF family. Vascular endothelial growth factor C (VEGF-C) maybe a target of anti-angiogenesis therapy for endometriosis [9]. However, the research of VEGF-C in endometriosis is still in the initial stage. Still, more experiments are urgently needed to distinguish the relationship between VEGF-C and endometriosis.

The common anti-angiogenic therapies of endometriosis is angiogenic growth factor inhibitors, Endogenous angiogenesis inhibitors (endostatin), Statins and so on [10]. Endostar is a new type of endothelin, has been developed as anti-neoplastic drug. Currently endostatin is consid-

**Table 1.** Characteristics of endometriosis patients and control group

| | Endometriosis (n=83) | Controls (n=30) |
|---|---|---|
| Age (y) | | |
| Mean (Std) | 39.23 (7.65) | 37.46 (9.37) |
| Median (Range) | 38 (21-56) | 38 (18-52) |
| Grade | | |
| I | 20 | N/A |
| II | 26 | N/A |
| III | 21 | N/A |
| IV | 16 | N/A |

ered to be an effective and broad-spectrum angiogenesis inhibitor. However, it has not been applied to the treatment of endometriosis.

Therefore, we conducted a study to investigate the relationship between VEGF-C and endometriosis. At the meantime, the role of VEGF-C in the pathogenesis of endometriosis is also investigated. An additional aim is evaluating the therapeutic effects of Endostar in the rat endometriosis model.

## Materials and methods

### *Patients*

A case-control study was conducted on women with and without endometriosis in the proliferative and secretory phases of the menstrual cycle. 83 patients with a surgery and histological diagnosis of endometriosis were selected. The subjects were 21 to 56 years old, had no other reproductive disorders or any tumors and had not been taking any hormone therapy for at least 3 months before sample collection. The stage of endometriosis was determined according to the classification of the American Society for Reproductive Medicine [11]. Medical history of all patients was collected, including CA125 values.

The control group consisted of 30 women of reproductive age without endometriosis, fibrosis, pelvic adhesions, or infertility, aged 18 to 52 years old. These women were subjected to surgery for simple ovarian cyst removal, with surgery confirmation of the absence of endometriotic lesions and had not been taking hormone medication for at least 3 months before biopsy collection. All tissue samples were obtained with informed consent and all procedures were performed in accordance with the Human Investigation Ethical Committee of Shanghai Jiaotong University Affiliated Sixth People Hospital South Campus.

### *Tissue microarrays*

Core tissue biopsy specimens (diameter 2 mm) were obtained from individual paraffin-embedded ectopic endometrial tissue (donor blocks) and arranged in new recipients paraffin blocks (tissue assay blocks). The control specimens were included in each of the assay blocks.

### *Immunohistochemistry*

Endometriosis tissue microarrays were incubated for 1 h at RT with the goat serum in order to block any nonspecific immunoassay. The primary antibody (monoclonal antibody DLK1 1:100) was incubated overnight at 4 degree centigrade. After rinsing with PBS, anti-rat immunoglobulin (IgG) was applied for 1 h. They were subsequently stained with DAB Kit. Scoring was conducted according to the ratio and intensity of positive-staining cells: 0-10% scored 0, 11-30% scored 1, 31-60% scored 2, 61-100% scored 3. Then scored 0-1 was designated as low expression and scored 2-3 as high expression.

### *Animals*

Twenty virgin, mature, female rats (weighing 200 g and aged approximately 2 months) were housed at Shanghai SLAC Laboratory Animal CO.LTD. All investigations rigorously complied with the national standards for the care and use of laboratory animals. Endometriosis was induced surgically by transplanting an autologous fragment of endometrial tissue onto the inner surface of the abdominal [12]. All procedures were carried out under sterile surgical conditions. After two weeks the rats underwent a second surgery to evaluate the size and viability of the ectopic endometrial tissue. All rats were successful modeling. Subsequently these animals were randomly allocated into treatment (n=5) and control groups (n=5). Starting on the first day after the second surgery, rats allocated to the treatment group received a daily intraperitoneal injection of Endostar (2 mg/kg/d) for 14 successive days. Rats allocated to the control group received an equal volume (40 mL) of PBS in a similar fashion. 2 weeks after the beginning of treatment (24 hours after the last dose of Endostar), all rats were killed and a third surgery was performed.

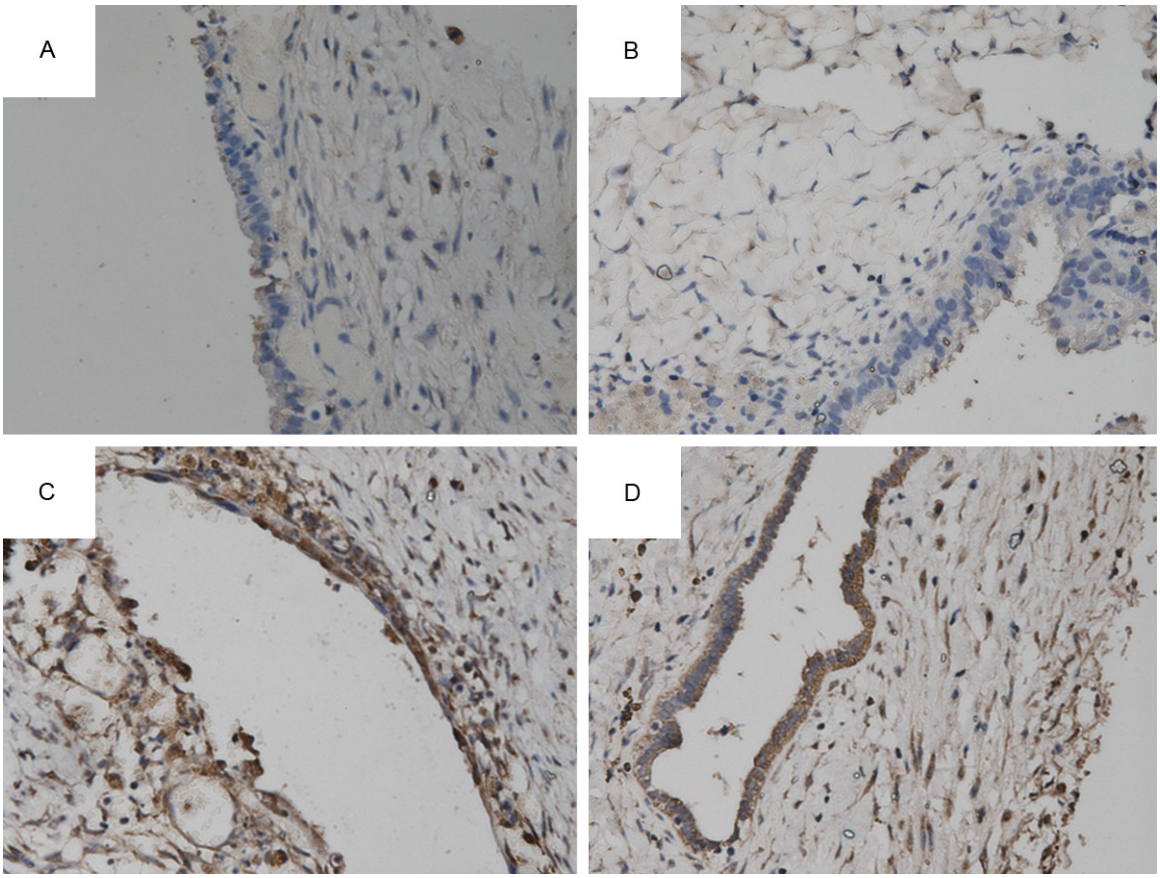


**Figure 1.** Immunohistochemical expression of VEGF-C in endometriotic lesions and control group. A, B. Low expression of VEGF-C in control group. C, D. High expression of VEGF-C (brown colour) in endometriotic lesions (original magnification ×400).

All viable implants were measured in three dimensions (length × width × height, in millimeters) and weight. Then the implants were immediately fixed in 10% buffered formalin for 24 hours. Further evaluating by HE staining and Immunohistochemistry were taken.

*Statistical analysis*

Data were presented as the means±standard error of the mean (SEM). Statistical analyses were done using SPSS 17.0 for windows (IBM). The chi-square test, or ANOVA were used for comparison between groups. Values of $P < 0.05$ were considered statistically significant.

## Results

*Expression of VEGF-C in endometriotic lesions*

The mean age of the 83 endometriosis patients is 39.23±7.65 (range 21-56 yr) while the 30 control patients is 37.46±9.37 (range 18-52 yr). The stage of endometriosis was determined according to the classification of the American Society for Reproductive Medicine [11]. 83 endometriosis patients staged as grade I (n=20), grade II (n=26), grade III (n=21), and grade IV (n=16) (**Table 1**), 36 proliferative and 47 secretory phase.

Immunohistochemical expression of VEGF-C was higher in endometriotic tissues than in control normal ovary tissues ($P < 0.01$). The high expression of VEGF-C in endometriotic tissues was 69.88% (58/83) while in control group was 10% (3/30) (**Figure 1**). 58.70% (27/46) lesions staged grade I-II were high VEGF-C expression and 83.78% (31/37) lesions staged grade III-IV were high VEGF-C expression. Expression of VEGF-C was higher in the endomertriosis grade III-IV than in endomertriosis grade I-II ($P$=0.013). Analyzing the relationship between VEGF-C expression and menstrual cycle, data showed that was no difference between two menstrual phases ($P$=0.374) as well as serum CA125 ($P$=0.188) (**Table 2**).

**Table 2.** Expression of VEGF-C in endometriosis and control group

| | VEGF-C High | VEGF-C Low | Total | $X^2$ | *P*-value |
|---|---|---|---|---|---|
| Controls | 3 | 27 | 30 | | < 0.0001* |
| Endometriosis | 58 | 25 | 83 | | |
| Grade | | | | | |
| I-II | 27 | 19 | 46 | 6.132 | 0.013 |
| III-IV | 31 | 6 | 37 | | |
| CA125 | | | | | |
| ≥ 35 IU/ml | 39 | 13 | 52 | 1.732 | 0.188 |
| < 35 IU/ml | 19 | 12 | 31 | | |
| Menstrual phase | | | | | |
| Proliferative phase | 27 | 9 | 36 | 0.792 | 0.374 |
| Secretory phase | 31 | 16 | 47 | | |

*Compared control group with endometriosis group $P < 0.0001$.

**Table 3.** Characteristics of Endostar treated group and control group

| | Weight ($\bar{X}$±S, g) | *P*-value | Volume ($\bar{X}$±S, $mm^3$) | *P*-value |
|---|---|---|---|---|
| Controls (n=5) | 0.1184±0.014291 | 0.001527 | 37.90±18.50 | 0.0435 |
| Treated (n=5) | 0.0568±0.020231 | | 8.57±3.10 | |

*Effects of Endostar on rat model*

No rat with nonviable implants was taken out of the study. Macroscopically, the implants appeared as cystic structures containing clear, serous fluid and were found to be well vascularized and attached to the abdominal wall peritoneum before randomly allocated into treatment and control groups. After two weeks of treatment we measured the volume and weight of all viable implants per rat. There was a significant reduction in the mean implant volume per rat in the treatment group (8.57±3.10 $mm^3$) as compared with the control group (37.90±18.50 $mm^3$) (*P*=0.0435). And also was a significant reduction in the mean implant weight per rat in the treatment group (0.0568 ± 0.020231 g) as compared with the control group (0.1184± 0.014291 g) (*P*=0.001527) (**Table 3**; **Figure 2**).

The HE staining of the specimens is shown in **Figures 3**, **4**. We observed ectopic endometrium was significantly reduced, glandular epithelial vacuolar degenerated, interstitial cells were small and sparse in treatment group. On the contrary, in control group we observed ectopic endometrial grew well, interstitial cells grew well, and the peripheral vasculars were rich. Immunohistochemical staining of the specimens for VEGF-C is shown in **Figures 3**, **4**. There was a significant reduction in VEGF-C expression in the treatment group as compared with the control group.

## Discussion

Endometriosis is a common chronic disease. Endometriosis is a benign disease but it shares several characteristics with invasive cancer. Its pathogenesis is still not clear. In recent years, studies have shown that angiogenesis is an important pathological process in the pathogenesis of endometriosis. Endometriotic lesions require neovascularization to deliver essential oxygen and nutrient supply for the development and progression of the disease [13]. Endometrial angiogenic disorders may be the basis of pathogenesis. Vascular endothelial growth factor (VEGF) is the most important factor in the regulation of both normal and abnormal angiogenesis [14, 15]. Angiogenesis induced by VEGF may be one of the most important pathogenesis of EMs, and closely related to vascular endothelial growth factor receptors 2 (VEGFR-2) [16]. Sung et al. studied VEGF in gene level. Following extraction of genomic DNA, genotyping of the -460 C/T and +405 C/G polymorphisms of the VEGF gene were performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis in their research. They found genotype distribution of the +405 C/G polymorphism was significantly different between patients with and without endometriosis. These findings suggest that the VEGF +405 C/G polymorphism may be associated with the risk of advanced stage endometriosis [17]. Vascular endothelial growth factor C (VEGF-C) is one of the members of the VEGF family [18, 19] (VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor PlGF). VEGF-C known as VEGF-related protein, was first identified in 1996 [20]. The encoded protein of VEGF-C can promote angiogenesis and endothelial cell growth, influence the permeability of blood vessels. VEGF-C has unique N-terminal and C-terminal extensions, which are not present in VEGF-A, VEGF-B or PlGF [21]. In our study, we

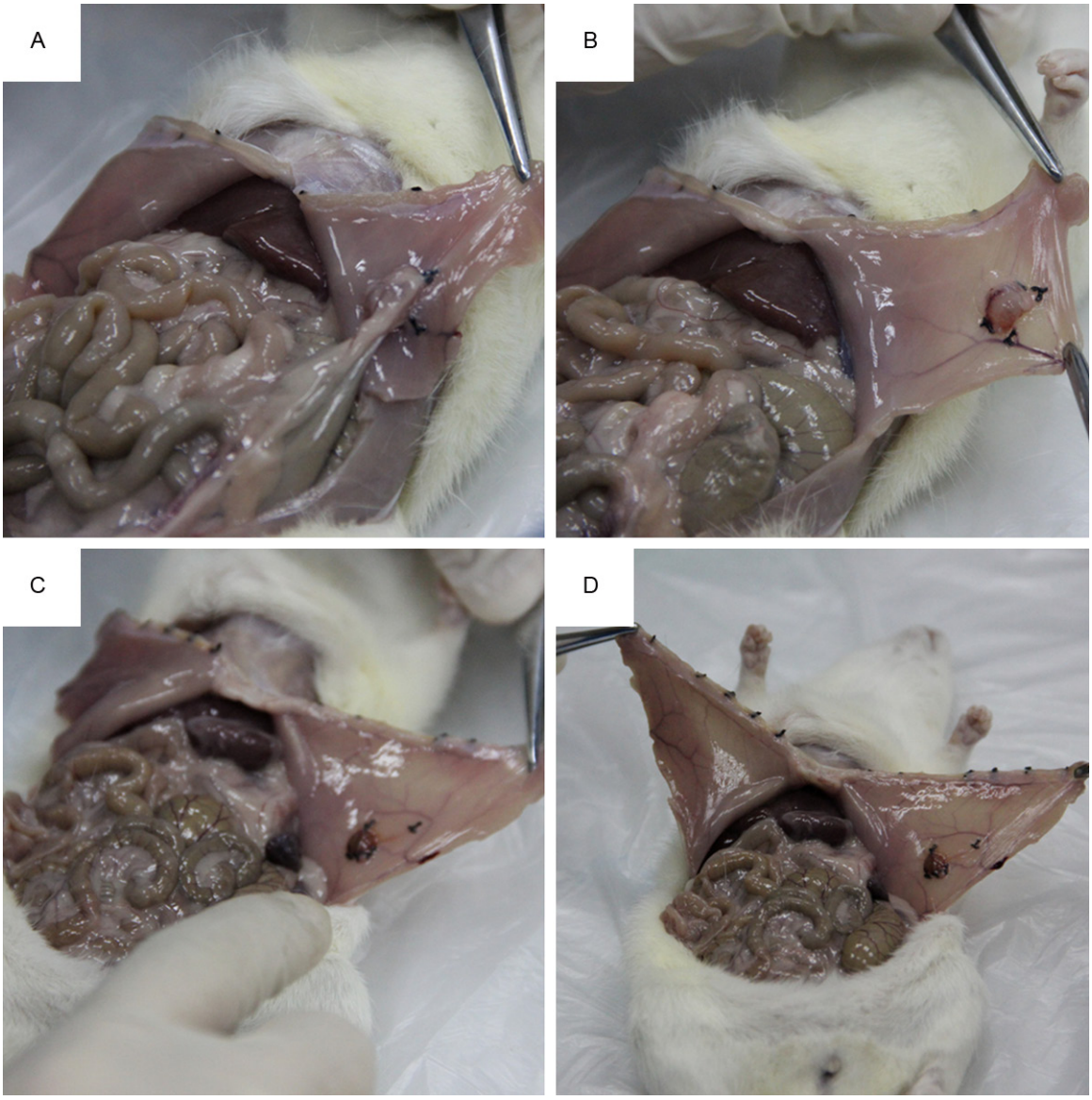


**Figure 2.** Observation of rat endometriosis model after treatment. A, B. In control group the implants appeared as cystic structures containing clear, serous fluid, well vascularized and attached to the abdominal wall peritoneum; C, D. In treatment group the implants and tissue adhesion reduced.

observed immunohistochemical expression of VEGF-C was higher in endometriotic tissues than in control normal ovary tissues ($P < 0.01$) and higher in the endomertriosis grade III-IV than in endomertriosis grade I-II ($P=0.013$). This suggests that VEGF-C is associated with endometriosis and the expression of VEGD-C has a relationship with the severity of endometriosis. The higher the grade of endomertriosis (III-IV), the more highly expressed of VEGF-C.

Serum CA125 levels may be elevated in endometriosis. However, compared to laparoscopy, measuring serum CA125 levels has no value as a diagnostic tool [22]. However, CA125 can be used to monitor the completeness of surgery [23]. In our study, we investigated the expression of VEGF-C in endometriosis patients with different CA125 value (< 35 IU/ml, ≥ 35 IU/ml). There was no difference between two groups ($P=0.188$). This indicates that the expression of VEGF-C and serum CA125 value has no correlation.

Current therapeutic options for endometriosis consist of various hormonal treatments aimed

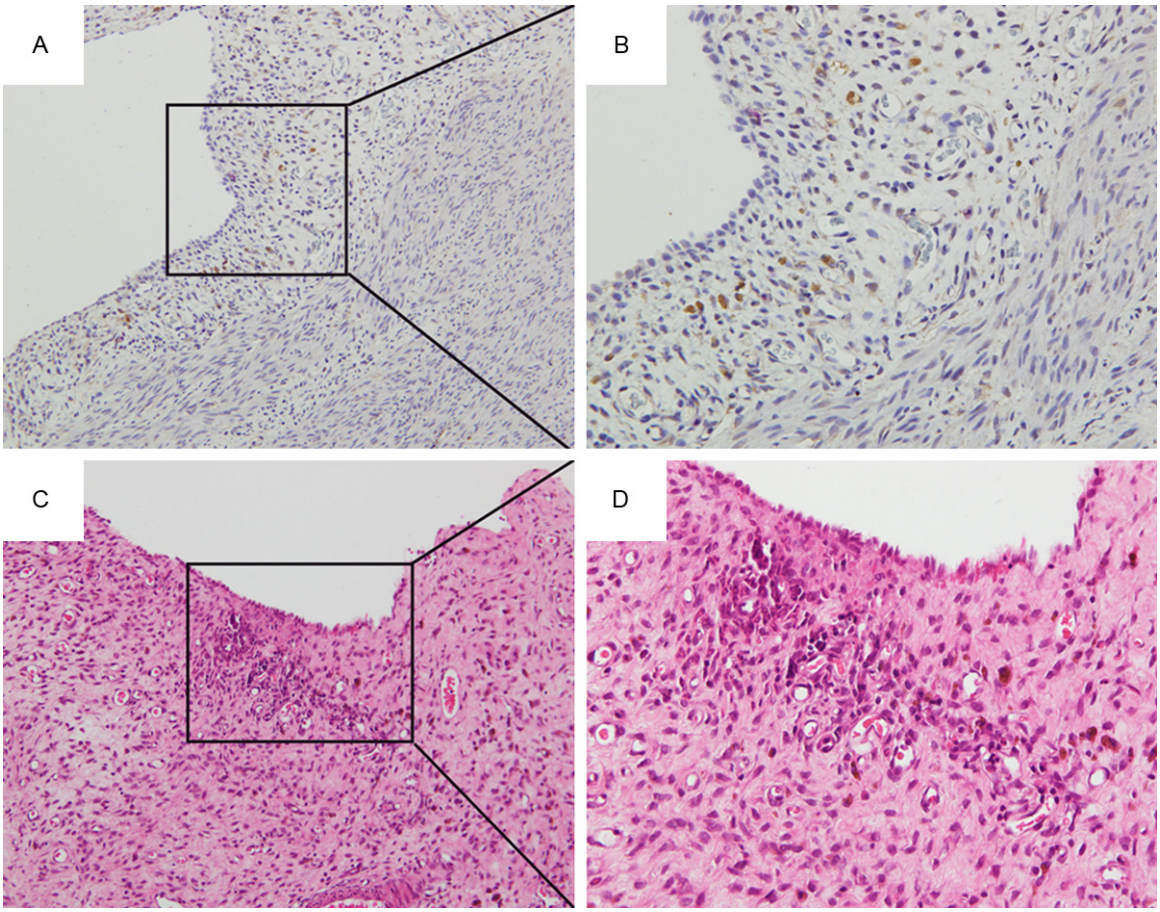


**Figure 3.** The HE staining and immunohistochemical staining of the implants from rat endometriosis mode in treated group. A. Overview, B. Low expression of VEGF-C In immunohistochemical staining (original magnification ×400); C. Overview; D. In HE staining, ectopic endometrium was significantly reduced, glandular epithelial vacuolar degenerated, interstitial cells were small and sparse (original magnification ×400).

at interrupting the cycles of stimulation and bleeding of endometriotic tissue. This treatment for endometriosis however is not very satisfactory [24]. It has the potential advantage of lower recurrence rates and less endocrine side effects than conventional surgical and hormonal therapies [25]. Anti-angiogenic drugs may become a new method of treatment of endometriosis. The common anti-angiogenic therapies of endometriosis are angiogenic growth factor inhibitors, Endogenous angiogenesis inhibitors (endostatin), Statins and so on [10]. Endostatin is an endogenous inhibitors of angiogenesis. It is derived from extracellular matrix molecules [26], which is formed in the body and have been shown to inhibit the development of new blood vessels [27]. Moreover, endostatin suppresses matrix metalloproteinase (MMP) -2, -9 and -13 activity and blocks the binding of VEGF to its receptor [28, 29]. Endostar is a new type of endothelin with higher stability, longer half-life, stronger biological activity. In this study, we treated the endometriosis model rat with Endostar. The result was a significant reduction in the mean implant volume and weight per rat in the treatment group as compared with the control group ($P < 0.05$). Immunohistochemical expression of VEGF-C also reduced in the treatment group. This shows that Endostar can effectively inhibit angiogenesis. Maybe it can be used in clinical treatment. However, clinical evidence for the efficacy of anti-angiogenic treatment is needed. On the other hand, VEGF-C maybe can monitor the efficacy of anti-angiogenic treatment.

In conclusion, VEGF-C may be involved in the pathogenesis of endomertriosis by regulating the angiogenesis. The expression of VEGF-C has a relationship with the severity of endometriosis. Endostar has therapeutic effects of

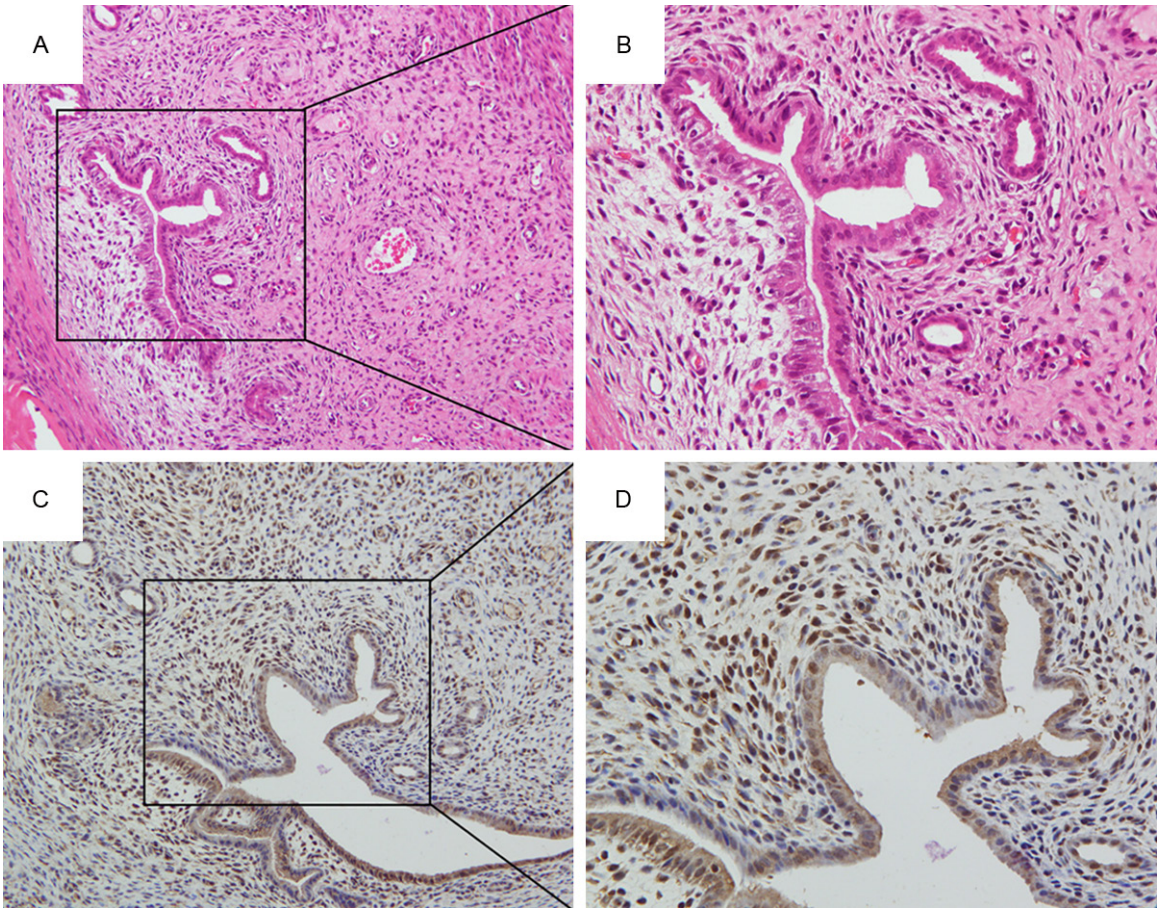


**Figure 4.** The HE staining and immunohistochemical staining of the implants from rat endometriosis mode in control group. A. Overview; B. In HE staining, ectopic endometrial grew well, interstitial cells grew well, and the peripheral vasculars were rich (original magnification ×400); C. Overview; D. High expression of VEGF-C (brown colour) in immunohistochemical staining (original magnification ×400).

endometriosis lesions in the rat endometriosis model. It may be a new direction for the treatment of endometriosis. At the meantime, VEGF-C maybe can monitor the efficacy of anti-angiogenic treatment.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Rong Zhang, Department of Obstetrics and Gynecology, Shanghai Jiaotong University Affiliated Sixth People Hospital South Campus, Shanghai 201499, P. R. China. Tel: +86-21-57421559; E-mail: rongzhang1965@163.com

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